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ORIGINAL ARTICLE

Anticancer Activity of Copper Complex of (4R)-(-)-2-Thioxo-4-thiazolidinecarboxylic Acid and 3-Rhodaninepropionic Acid on Prostate and Breast Cancer Cells by Fluorescent Microscopic Imaging

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Abstract Copper complexes with strong anticancer activity are promising new drugs for treatment of patients with metastatic cancer. Copper 8-hydroxyquinoline-2-carboxaldehyde-thiosemicarbazide (CuHQTS) and copper 8-hydroxyquinoline-2-carboxaldehyde-4,4-dimethyl-3-thiosemicarbazide (CuHQDMTS) were found to have strong anticancer activity against cisplatin-resistant neuroblastoma cells and prostate cancer cells. This study aimed to synthesize and characterize two new anticancer copper complexes, copper complex of (4*R*)-(−)-2-Thioxo-4-thiazolidinecarboxylic acid (CuTTDC), and copper complex of 3-Rhodaninepropionic acid-copper complex (CuRDPA). Cell growth inhibition and cytotoxicity of CuTTDC and CuRDPA on prostate and breast cancer cells were evaluated with Cell Counting Kits-8 (CCK8) assay and fluorescent microscopic imaging respectively. Strong anticancer activity of CuTTDC and CuRDPA was demonstrated by growth inhibition and cytotoxicity of prostate and breast cancer cells treated with these two copper complexes, supporting further investigation of potential use of these two new anticancer complexes for treatment of prostate and breast cancer metastasis.

Keywords Anticancer copper complex · (4R)-(-)-2-Thioxo-4-thiazolidinecarboxylic acid · 3-Rhodaninepropionic acid · Prostate cancer · Breast cancer · Green fluorescence protein · Fluorescent microscopic imaging

Introduction

Advances in diagnostic imaging and treatment have significantly improved survival of the patients diagnosed with early stage prostate or breast cancer, but overall survival of the patients diagnosed with advanced stage of prostate or breast cancers remains poor, particularly those patients with metastatic cancer refractory to anticancer drugs currently available [1–3]. Metallotherapeutic drugs, such as cisplatin and carboplatin, were widely used for cancer chemotherapy [4–7]. However, undesirable side effects and acquired resistance to cisplatin or other platinum anticancer drugs hampered clinical use of these metallotherapeutic anticancer drugs [8, 9]. There were continued efforts to develop new metallotherapeutic drugs, such as copper complexes, with strong anticancer activity for treatment of drug resistant cancer metastasis [10–14].

In an effort to develop anticancer agents for treatment of neuroblastoma, we found that copper complexes of pyrrolidine dithiocarbamate ($Cu(PDTC)_2$) had strong anticancer activity on cisplatin-resistant neuroblastoma cells [15]. We also synthesized and demonstrated strong anticancer activity of two copper thiosemicarbazone complexes, copper 8-hydroxyquinoline-2-carboxaldehyde – 4, 4-dimethyl-3-thiosemicarbazide complexes (CuHQDMTS) and copper 8-hydroxyquinoline-2-carboxaldehyde-thiosemicarbazide complex (CuHQTS), on cisplatin-resistant neuroblastoma cells [16]. More recently, CuHQTS and CuHQDMTS were

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found to have broad anti-prostate cancer activity as demonstrated by cytotoxicity of these copper complexes on GFP-tagged prostate cancer cells by fluorescent microscopic imaging [17]. Continuing from our efforts in developing new copper-based anticancer agents, we synthesized two copper complexes of (4*R*)-(−)-2-Thioxo-4-thiazolidinecarboxylic acid (TTDC) and 3-Rhodaninepropionic acid (RDPA) (Fig. 1) and tested their anticancer activity on prostate and breast cancer cells. TTDC is a chemical with sulfur atoms as potential donor atoms to coordinate copper, and presence of amino and carboxyl can provide flexible groups to connect with functional groups on a delivery vehicle for targeted drug delivery in vivo.

RDPA contains similar sulfur atoms and amino and carboxyl groups for copper coordination, which was reported to have phytogrowth inhibition activity in plants [18]. Based on chemical structure of these two molecules, we hypothesized that copper complex of TTDC and copper complex of RDPA might have strong anticancer activity against prostate and breast cancer cells. To test our hypothesis, we prepared copper complexes of TTDC and RDPA ligands (CuTTDC and CuRDPA), and tested their cell growth inhibition activity and cytotoxicity on prostate and breast cancer cells using CCK-8 assay and fluorescent microscopic imaging.

Materials and Methods

Chemicals, Reagents, and Cells

(4R)-(-)-2-Thioxo-4-thiazolidinecarboxylic acid (TTDC), 3-Rhodaninepropionic acid (RDPA) and $CuCl_2{\cdot}2H_2O$ were purchased from Sigma–Aldrich (St. Louis, MO). MSCV-Luciferase-EF1a-copGFP-T2A-Puro Pre-packaged Virus, a lentivirus containing a GFP and luciferase reporter gene, was purchased from System Biosciences (Palo Alto, CA). Prostate cancer cells (PC-3 and DU145) and breast cancer cell (SKBR3 and MD-MBA-231) were purchased from ATCC and cultured in RPMI 1640 (Sigma–Aldrich, St. Louis, MO) or Dulbecco's Modified Eagle Medium (DMEM, Sigma–Aldrich, St. Louis, MO) medium containing 10% fetal bovine serum (Sigma–Aldrich, St. Louis, MO) and 1% penicillin and streptomycin (Gibco, Auckland, NZ). IR spectra were recorded from 4000 to 400 cm^{-1} as KBr pellets on a Tensor 27 FT-IR spectrophotometer. ESI mass spectra were measured in a triple quadrupole Micromass QuattroLC spectrometer with an electroscopy/APCI source.

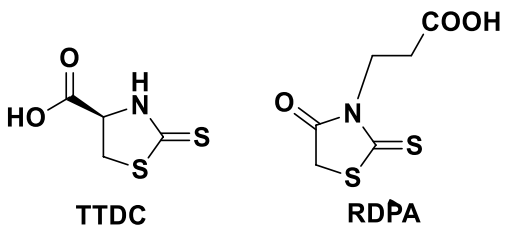


Fig. 1 Schematic presentation of chemical structure of (4*R*)-(−)-2-Thioxo-4-thiazolidinecarboxylic acid (TTDC) and 3-Rhodaninepropionic acid (RDPA) ligand

Preparation of TTDC and RDPA Copper Complexes

For preparation of CuTTDC complexes, a solution of $CuCl_2{\cdot}2H_2O$ (0.372 g, 2 mmol) in water (20 mL) was added dropwise to a stirred solution of TTDC (0.163 g, 1 mmol) in warm water (30 mL). The pH value of this solution was gradually adjusted to 7.0 with 0.1 M NaOH. The resulting mixture was refluxed for 3 h and then allowed to cool to room temperature with continuous stirring overnight. The volume of solvent was reduced and the blue-green precipitate was formed. The precipitate was collected with filtration, washed with diethyl ether. CuRDPA complexes were prepared in a method same as that described above. RDPA (0.208 g, 1 mmol) and $CuCl_2{\cdot}2H_2O$ (0.513 g, 3 mmol) were reacted to prepare orange CuRDPA. For CuTTDC, ESI-MS (methanol, m/z): 387.9, calcd for $C_8H_8N_2O_4S_4Cu$ $[M+H^+]$, found 387.8. IR (KBr, ν/cm^{-1}): 3480m, 1730s, 1552s, 1473s, 1214m, 1043m, 749m, 669s. For CuRDPA, ESI-MS (methanol, m/z): 470.9 calcd for $C_{12}H_{12}N_2O_6S_4Cu$ $[M+H^+]$, found 471.0 (M^+). IR (KBr, ν/cm^{-1}): 3454m, 1695s, 1505s, 1335s, 1277m, 1018m, 780s, 749m.

Preparation of GFP Cells

Prostate cancer cells carrying GFP reporter gene (GFP-PC-3 cells, and GFP-DU145 cells) were prepared as previously described [17]. In addition, breast cancer cells carrying GFP reporter gene (GFP-SKBR3, and GFP-MBA-MD-231 cells) were also established with lentiviral GFP vector in a method same as that described for preparation of GFP-carrying prostate cancer cells [17]. Briefly, these cell lines were established by infection of these cells with MSCV-Luciferase-EF1a-copGFP-T2A-Puro Lenti-virus in puromycin selection medium with a protocol from the Vendor (System Biosciences, Palo Alto, CA). After establishment of these GFP carrying cells, GFP expression was evaluated by fluorescent microscopic imaging using Olympus phase contrast microscope equipped with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI) and Olympus U-RFL-T-200 Power Supply.

CCK8 Cell Growth Inhibition Assay

Cell growth inhibitory effect of the CuTTDC and CuRDPA was determined by Cell Counting Kits-8 (CCK8) assay in a method from the vendor (Dojindo Laboratories, Kumamoto,

Table 1 Cell growth inhibition activity (IC_{50}) of CuTTDC and CuRDPA on prostate and breast cancer cells determined by CCK8 assay

	CuTTDC	CuRDPA	TTDC	RDPA
PC-3	42.57 ± 3.06[a]	2.40 ± 1.07	> 200	> 200
DU 145	57.64 ± 6.87	4.19 ± 1.50	> 200	> 200
MDA-MB-231	68.56 ± 2.28	14.40 ± 1.06	> 200	> 200
SKBR3	72.69 ± 2.08	18.87 ± 1.73	> 200	> 200

[a]μM, mean ± standard deviation (SD)

Japan). Briefly, cells growing in the logarithmic phase were seeded into a 96-well microplate (1×10^4 cells/well) 12 h prior to treatment with the copper complexes. The compounds of different concentrations dissolved in medium containing 0.1% DMSO was added to the wells, and incubated at 37 °C for 24 h. Aliquots of 5 μL of CCK8 solution were then added to each well. After incubation at 37 °C for 4 h, the absorbance of the color products was recorded at 460 nm with a microplate photometer (Thermo Fisher Scientific, Vantaa, Finland). The growth inhibition was calculated by dividing the average absorbance of the cells treated with a copper complex or ligand by that of the control. The inhibition concentration 50% (IC_{50}) values were calculated by SPSS.

Cytopathological Effects by Microscopic Examination

Prostate and breast cancer cells were treated with various concentrations of TTDC, RDPA, CuTTDC, CuRDPA for noted time and evaluated for morphological cytopathological effects with an Olympus phase contrast microscope equipped with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI). Spherical morphology and detachment of the treated tumor cells indicated cyopathological effects induced by the compounds.

Fluorescent Microscopic Imaging of Cytotoxicity on GFP-PC-3 Cells

Cancer cells carrying GFP reporter gene (GFP-PC-3, GFP-DU145, GFP-SKBR3, and GFP-MBA-MD-231cells) were treated with TTDC, RDPA, CuTTDC, CuRDPA of different concentrations for noted time. Effect of these drugs on GFP signal and cell morphology were examined and recorded by fluorescent imaging using a fluorescence microscope (400× magnification) as previously described [17].

Statistical Analysis

The data was expressed as mean ± SD. Means were compared using Student's *t* test. A P value < 0.05 was considered significant.

Results

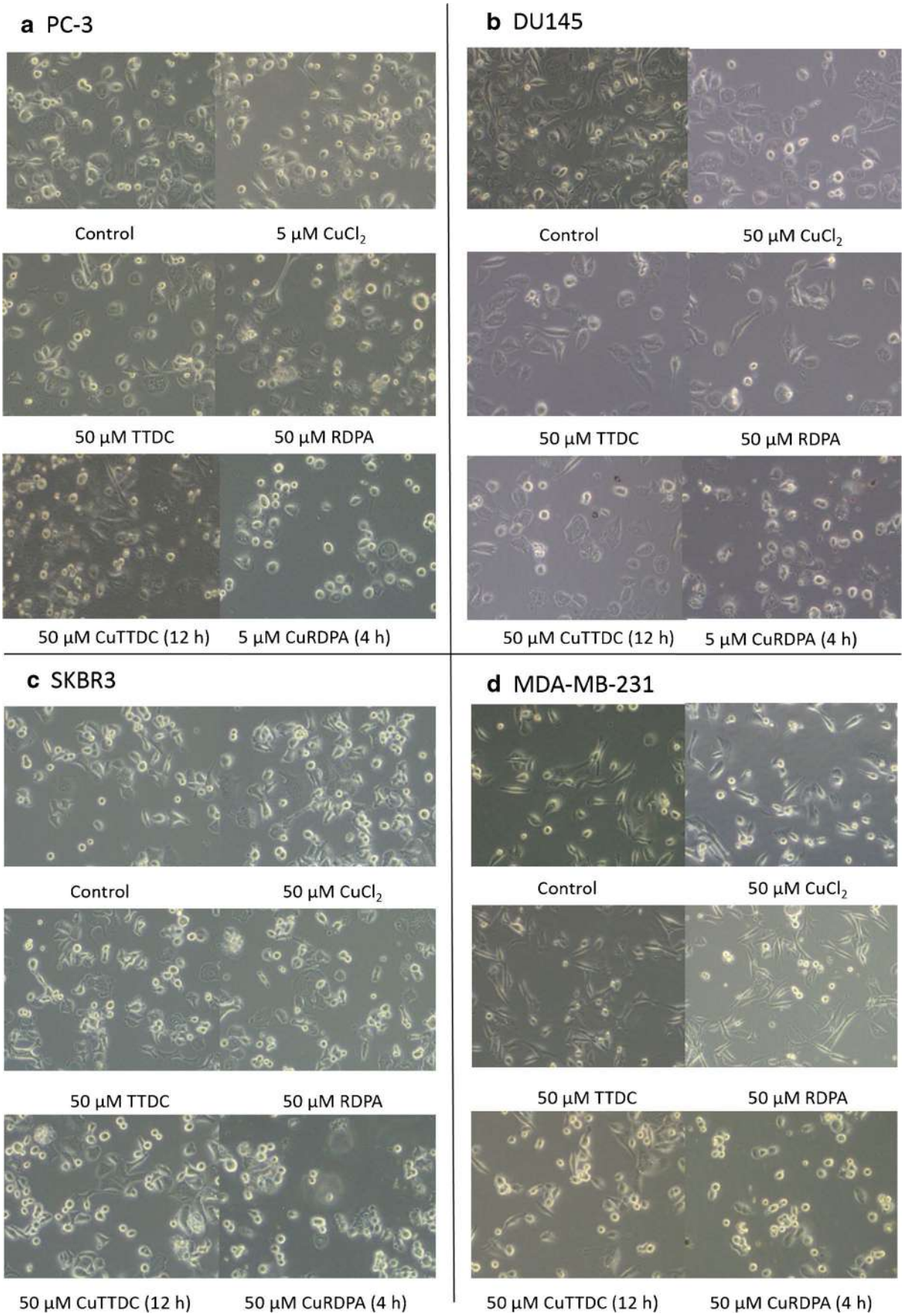
Preparation of TTDC and RDPA Copper Complexes

A solution of $CuCl_2 \cdot 2H_2O$ dissolved in water was added dropwise to a stirred water solution of TTDC to obtain CuTTDC. CuRDPA complex was prepared in a similar fashion as described for preparation of CuTTDC. These two new copper complexes have revealed well-defined molecular vibrations in IR region. The high pH and high metal reaction ratio allow ligands to deprotonate adequately and to bind enough copper ions by donor atoms. Significant changes between ligands and copper compound were observed in IR spectra. The characteristic four thioamide bands at 1500, 1200–1300 cm^{-1} attributed to ν (CN) vibrations (thioamide I and II bands) and at 1000, 750–650 cm^{-1} attributed to ν (CS) vibrations (thioamide III and IV bands) are perturbed to a greater or less extent. The perturbation most significant is shown in thioamide III and IV bands. The downfield shifts are

Table 2 Effects of TTDC or RDPA ligands on prostate and breast cancer cell proliferation by CCK8 assay

	TTDC		RDPA	
	100 μM	200 μM	100 μM	200 μM
PC-3	101.5 ± 4.05[a]	103.1 ± 6.19	103.5 ± 2.59	95.9 ± 3.27
DU 145	98.6 ± 5.61	100.0 ± 6.29	98.9 ± 4.21	89.8 ± 2.49
MDA-MB-231	98.5 ± 11.3	103.1 ± 7.91	98.1 ± 7.58	101.0 ± 5.39
SKBR3	83.6 ± 5.87	76.7 ± 5.26	81.4 ± 16.2	82.9 ± 9.28

[a]% of cancer cells treated with 100 or 200 μM of TTDC or RDPA for 24 h (mean ± SD %) relative to control cancer cells without TTDC or RDPA treatment


a PC-3
Control
5 μM CuCl2
50 μM TTDC
50 μM RDPA
50 μM CuTTDC (12 h)
5 μM CuRDPA (4 h)
b DU145
Control
50 μM CuCl2
50 μM TTDC
50 μM RDPA
50 μM CuTTDC (12 h)
5 μM CuRDPA (4 h)
c SKBR3
Control
50 μM CuCl2
50 μM TTDC
50 μM RDPA
50 μM CuTTDC (12 h)
50 μM CuRDPA (4 h)
d MDA-MB-231
Control
50 μM CuCl2
50 μM TTDC
50 μM RDPA
50 μM CuTTDC (12 h)
50 μM CuRDPA (4 h)

◂**Fig. 2** Cytopathological effects (CPE) of CuTTDC and CuRDPA on PC-3 (**a**) and DU145 (**b**) prostate cancer cells, and SKBR3 (**c**) and MDA-MB-231 (**d**) breast cancer cells. Significant CPE (shrinkage, spherical morphology and detachment of the cells) were observed on the cells treated with 50 μM CuTTDC for 12 h or 50 μM CuRDPA for 4 h respectively, but not on the control cells treated with an equal amount of solvent (0.05% DMSO) or 50 μM of $CuCl_2$, TTDC, RDPA ligand up to 24 h

observed from 1053 cm^{-1} and 740 cm^{-1} in the free TTDC to 1043 cm^{-1} and 669 cm^{-1} in the CuTTDC, and from 1062 cm^{-1} and 768 cm^{-1} in the free RDPA to 1018 cm^{-1} and 749 cm^{-1} in the CuRDPA, indicating exocyclic thione sulfur atom chelating copper ions. No band at 3100 cm^{-1} due to ν (NH) vibration is observed showing that deprotonation has occurred. The shifts in the thioamide bands and carboxyl bands indicate that donor atoms are involved in coordination to copper ions. The results of ESI-MS analysis indicated that CuTTDC and CuRDPA existed as 1:2 chelating mode (copper: ligand), and no chloride ions or water molecules were appended to the final copper complexes.

Inhibition of Cancer Cell Proliferation

Cell growth inhibition activity of CuTTDC and CuRDPA was tested by CCK-8 assay in a method as described previously [17, 19]. Proliferation of prostate cancer cells was suppressed by treatment of the cells with CuTTDC or CuRDPA (Table 1). CuRDPA exhibited stronger cell growth inhibition activity than cell growth inhibition activity of CuTTDC. Both CuRDPA and CuTTDC displayed a stronger cell growth inhibition activity on prostate cancer cells (IC_{50} at 2.40 ± 1.07 μM on PC3 cells and IC_{50} at 4.19 ± 1.50 μM on DU145 cells) than their cell growth inhibition activity on breast cancer cells (IC_{50} at 4.40 ± 1.06 μM on MDA-MB-231 cells and IC_{50} at 18.87 ± 1.73 μM on SKBR3 cells).

In control, TTDC and RDPA ligands exhibited minimal cell growth inhibition activity on prostate or breast cancer cells when the cells were treated with a large dose (100 and 200 μM) of TTDC or RDPA ligand respectively (Table 2).

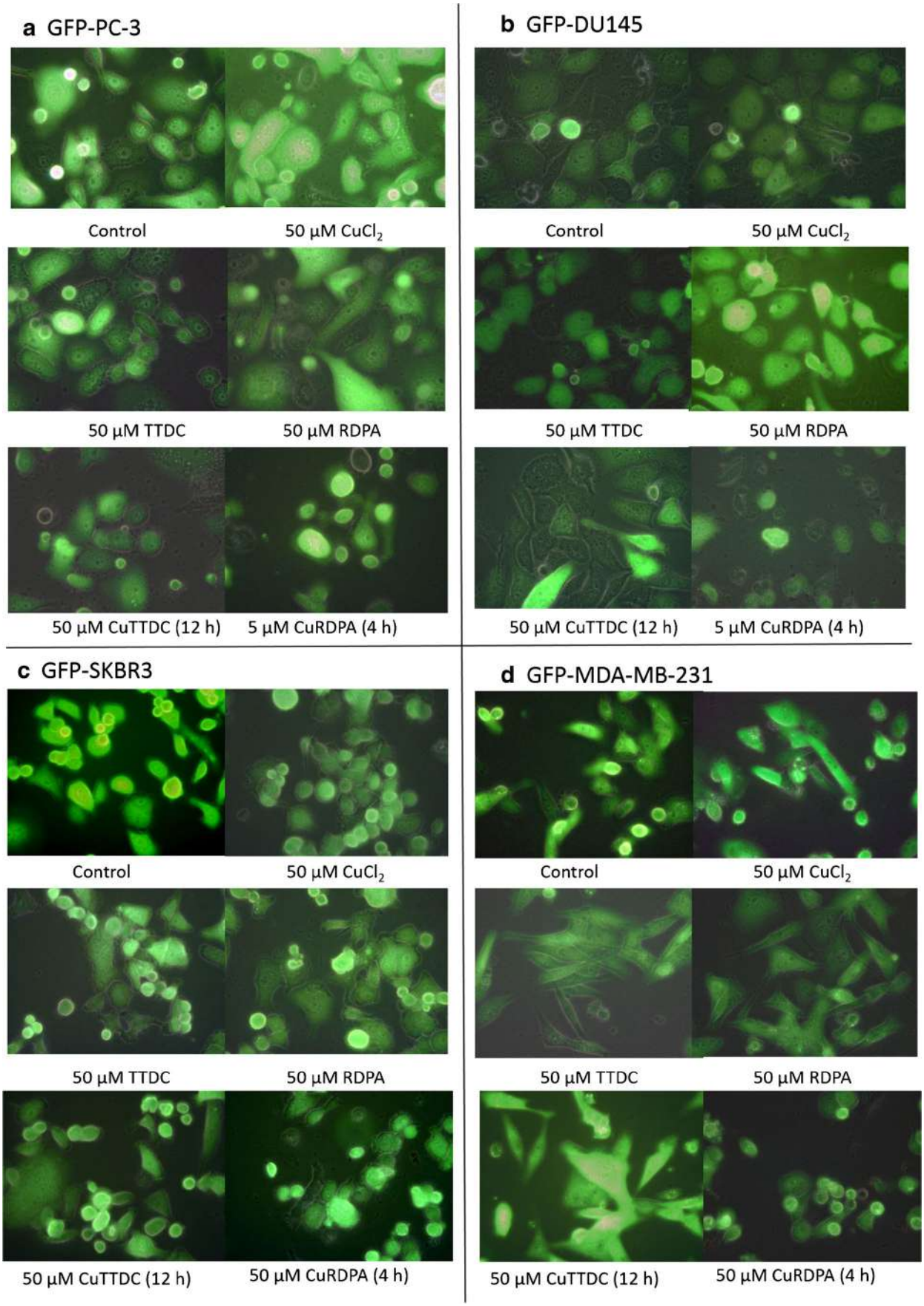
In comparison to growth of untreated prostate cancer cells, no significant cell growth inhibition was detected when prostate cancer cells were treated with TTDC or RDPA ligands except mildly suppressed proliferation of DU145 prostate cancer cell treated with 200 μM of RDPA ligand ($89.8 \pm 2.49\%$, $P < 0.01$). No significant cell growth inhibition was detected when MDA-MB-231 breast cancer cells were treated with TTDC or RDPA ligands, but mild cell growth inhibition was detected when SKBR3 breast cancer cells were treated with TTDC ($76.7 \pm 5.26\%$, $p < 0.01$) or RDPA ($82.9 \pm 9.28\%$, $p < 0.03$).

Cytopathological Effects of Cancer Cells Treated with Copper Complexes

In addition to cell growth inhibition assay, we evaluated cytotoxicity of CuTTDC and CuRDPA on prostate and breast cancer cells by microscopic examination of cytopathological effects of the cells treated with these two copper complexes. Significant cytopathological changes of prostate cancer cells, such as spherical morphology and shrinkages, were observed as early as 4 h after treatment of the cells with 5 μM of CuRDPA or 50 μM of CuTTDC for 12 h (Fig. 2a, b). Cytopathological changes of breast cancer cells were observed after treatment with 5 μM of CuRDPA for 4 h or 50 μM of CuTTDC for 12 h as well (Fig. 2c, d). These results suggested different strength of anticancer activity of CuRDPA and CuTTDC on these cancer cell lines. In contrast, no cytopathological changes were detected after treatment of cancer cells with 50 μM of TTDC, RDPA, or $CuCl_2$ for 24 h individually.

Cytotoxicity on GFP-Carrying Cancer Cells by Fluorescent Microscopic Imaging

Moreover, we examined anticancer activity of CuRDPA and CuTTDC on prostate and breast cancer cells carrying GFP reporter gene using fluorescent microscopic imaging [17]. The prostate cancer cells carrying GFP (GFP-PC-3 and GFP-DU145) and breast cancer cells carrying GFP reporter gene (and GFP-MDA-MB-231, GFP-SKBR3 cells) were treated with CuTTDC or CuRDPA of different concentrations, and effect of treatment on the cells were examined by fluorescent microscopic imaging using a fluorescent microscope. These cells carrying GFP reporter gene (GFP-PC3, GFP-DU145 and GFP-MDA-MB-231, GFP-SKBR3 cells) were established in a protocol modified from a method reported previously [20]. The GFP-PC-3, GFP-DU145, GFP-MDA-MB-231 and GFP-SKBR3 cells were treated with CuTTDC (50 μM) for 12 h, or CuRDPA (5 μM for prostate cancer cells or 50 μM for breast cancer cells) for 4 h, while control cells were treated with $CuCl_2$ (50 μM), TTDC (50 μM), or RDPA (50 μM) for 24 h. There was reduction of GFP fluorescent signals associated with cell shrinkage of GFP-PC-3 and GFP-DU145 cells following treatment of the cells with 50 μM of CuTTDC for 12 h or 5 μM of CuRDPA for 4 h. The similar therapeutic effects (reduction of fluorescent signals and cell shrinkage) were detected on the GFP-MDA-MB-231 and GFP-SKBR3 cells treated with 50 μM of CuTTDC for 12 h, or 50 μM of CuRDPA for 4 h. In contrast, no reduction of fluorescent signals or morphological changes of GFP-PC-3 and GFP-DU145 cells were detected after treatment of the cells with 50 μM of $CuCl_2$, TTDC, or RDPA for 24 h respectively (Fig. 3).


a GFP-PC-3
Control
50 μM $CuCl_2$
50 μM TTDC
50 μM RDPA
50 μM CuTTDC (12 h)
5 μM CuRDPA (4 h)
b GFP-DU145
Control
50 μM $CuCl_2$
50 μM TTDC
50 μM RDPA
50 μM CuTTDC (12 h)
5 μM CuRDPA (4 h)
c GFP-SKBR3
Control
50 μM $CuCl_2$
50 μM TTDC
50 μM RDPA
50 μM CuTTDC (12 h)
50 μM CuRDPA (4 h)
d GFP-MDA-MB-231
Control
50 μM $CuCl_2$
50 μM TTDC
50 μM RDPA
50 μM CuTTDC (12 h)
50 μM CuRDPA (4 h)

◂**Fig. 3** Therapeutic effects of CuTTDC and CuRDPA on prostate cancer cells carrying GFP reporter gene (**a** and **b**, prostate cancer GFP-PC-3 cells and GFP-DU145 cells) and breast cancer cells carrying GFP reporter gene (**c** and **d**, breast cancer GFP-SKBR3 cells and GFP- MDA-MB-231) by fluorescent microscopic imaging. The prostate cancer cells were treated with CuTTDC (50 μM, for 12 h) or CuRDPA (5 μM, for 12 h), while breast cancer cells were treated with CuTTDC (50 μM, for 12 h) or CuRDPA (50 μM, 4 h), compared to control cells treated with 50 μM of TTDC or RDPA ligand or $CuCl_2$ for 24 h. Significant change of cell morphology and reduction of florescent signal changes were observed on the cells treated with CuTTDC and CuRDPA, but not on control cells treated with an equal amount of solvent (0.05% DMSO), 50 μM of $CuCl_2$, TTDC, or RDPA ligands for 24 h

Discussions

Strong anticancer activity of CuTTDC and CuRDPA on prostate and breast cancer cells was demonstrated by cell growth inhibition assay and microscopic imaging. Molecular mechanism of anticancer activity of CuTTDC and CuRDPA remains to be elucidated, which may be related to oxidative stress and DNA damage of the cells treated with these two copper complexes. We found that CuRDPA had stronger anticancer activity than CuTTDC on prostate and breast cancer cells, likely related to different functional groups on these complexes, an extra carbonyl group may contribute to stronger anticancer activity of CuRDPA than anticancer activity of CuTTDC. It was also noticed that cell growth inhibition activity of CuRDPA on prostate cancer cells was stronger than its cell growth inhibition activity on breast cancer cells, although the reasons were still unclear.

Additional studies are necessary to evaluate cytotoxicity of CuTTDC and CuRDPA on non-malignant proliferating cells. Numerous copper complexes were found to have strong anticancer activity both in vitro and in vivo [10, 11]. However, non-specific cytotoxic on non-malignant proliferating cells often limits clinical use of anticancer copper complexes due to side effects associated with their toxicity to normal organs or tissues in humans [11]. In addition to continued efforts to search for cancer-specific copper complexes as matallotherapeutic drugs, one of promising approaches to improve cancer specificity of anticancer copper complexes is targeted delivery in vivo using a tumor-targeting delivery vehicle. Copper conjugates containing an active group, such as amino or carboxylic acid groups, are candidates for targeted drug delivery. Copper-sulfur based complexes with strong anticancer activity such as Metal-dithiocarbamate compounds are also desirable for targeted delivery [10, 16]. Sulfur and amino groups in CuTTDC and CuRDPA make it feasible to attach them to delivery vehicle such as peptide or monoclonal antibody for targeted delivery. Furthermore, radioactive copper isotopes such as positron emitting ^{64}Cu radionuclide, may be used for radiolabeling CuTTDC or CuRDPA conjugates and tracking targeted delivery in vivo with positron emission tomography (PET) imaging [21–23].

Conclusion

In conclusion, we demonstrated cell growth inhibition and cytotoxicity of two new copper complexes, CuTTDC and CuRDPA, on prostate and breast cancer cells. CuTTDC and CuRDPA exhibited variable strength of anticancer activity, likely related to different functional groups on TTDC and RDPA ligands. The findings of current study invite further investigation of anticancer activity of CuTTDC and CuRDPA on other cancer cells and targeted delivery of these two anticancer copper complexes for treatment of prostate and breast cancer metastasis.

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Compliance with Ethical Standards

Conflict of Interest There are no conflicts of interest.

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